IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Yan

Serial No.: 09/997,240

Filed: November 30, 2001

For: Isolated Homozygous Stem Cells, Differentiated Cells Derived Therefrom, And Materials And Methods For Making And Using Same.

Art Unit: To Be Assigned

Examiner: To Be Assigned

Atty. Docket: 0249-0001

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicant(s) herewith respectfully requests the following amendments:

IN THE SPECIFICATION:

1. On page 2, following the first paragraph and before the section entitled Field of the Invention, please insert the following paragraph:

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

2. In section II, titled **Background of the Invention and Description of Related Art**, please replace page 2, 3rd complete paragraph with the following:

In 1981, Evans and Kaufman described the technique for isolating embryonic stem (ES) cell lines from mouse blastocysts. Evans et al., "*Establishment in Culture of Pluripotent Cells from Mouse Embryos,*" Nature **292**:154-6 (1981). In this procedure, the inner cell mass (ICM) was used to give rise to a cell line that remained undifferentiated and pluripotent, i.e., the cells had the capacity to develop into any cell type. ES cell lines were subsequently produced in other animal models including chicken (Pain et al, Development **122**:2339-48 (1996)), hamster (Doetschmann et al., Dev. Biol. **127**:224-7 (1988)), swine (Wheeler et al., Reprod. Fertil. Dev. **6**:563-8 (1994)), marmoset (Thompson et al., Biol. Reprod. **55**:254-9 (1996)), and rhesus monkey (Thompson et al., Proc. Natl. Acad. Sci. USA **92**:7844-8 (1995)).

3. In section II, titled **Background of the Invention and Description of Related Art** please replace page 5, 4th complete paragraph with the following:

The development of other sources of pluripotent cells is hence needed in the art. The present invention provides one such source. In one embodiment, the present invention provides isolated homozygous stem (HS) cells that are isolated from a blastocyst-like mass that is created by: (a) fusing two oocytes or two spermatids; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte. Additionally, screening for stem cells that are homozygous is performed using genotyping when methods (a) or (d) are used.

4. In section II, titled **Background of the Invention and Description of Related Art** please replace page 6, 1st complete paragraph with the following:

The HS cells of the present invention are pluripotent, and raise no ethical concerns as they are isolated from cell-masses that are non-fertilized, and incapable of developing into viable embryos. Moreover, immunohistocompatibility matching is difficult to accomplish when heterozygous ES cell lines are employed in tissue or cell transplantation therapy, or maintained in banks and/or depositories. This is because the ES cell lines,

including those developed by Advanced Cell Technology and other organizations, are derived from fertilized embryos or from nuclear transfer techniques using adult differentiated cells, and are genomically heterozygous. Because the pluripotent stem cells of the present invention are homozygous (with minimal heterozygosity or uniform homozygosity), such cells may be used to overcome immunohistocompatibility problems faced by currently available transplantation, cell replacement, and gene therapy techniques employing ES cell lines, or maintaining ES cell line banks and/or depositories.

5. In section II, titled **Background of the Invention and Description of Related Art** please replace page 6, 3rd complete paragraph with the followoing:

Upon proper activation, a metaphase II oocyte can proceed to complete meiosis by the extrusion of one chromatid (i.e. the secondary polar body) and give rise to a haploid cell. Such meiosis-completed haploid oocyte self-replicates without cytokinesis, rendering it diploid and uniformly homozygous. Such meiosis-completed haploid oocytes, hence, may also be used to create the homozygous stem cells of the present invention with no heterozygosity. See also, Kaufman M.H., Robertson E.J., Handyside A.H., Evans M.J., "*Establishment of pluripotential cell lines from haploid mouse embryos*," J. Embryol. Exp. Morphol., 73:249-61 (1983).

6. In section II, titled **Background of the Invention and Description of Related Art** please replace page 8, 2nd complete paragraph with the following:

Naturally occurring spontaneous teratomas are diploid and occasionally polyploid (Surti *et al.,* Am. J. Hum. Gene. 47:635-643 (1990)). It is believed that diploid teratomous tissue occurs secondary to meiosis I, or due to fusion of the second polar body with the ovum (Eppig and Eicher, Genetics, 103:797-812 (1983); Eppig and Eicher, J. Hered., 79:425-429 (1988)). Further, teratomas have been proven to be genetically homozygous in heterozygous hosts (Linder, Proc. Natl. Acad. Sci. USA, 63:699-704 (1969); Linder and Power, Ann. Hum. Genet. 34:21-30, (1970); Linder et al., Nature, 254:597-598 (1975); Kaiser-McCaw et al., Cytogenet. Cell. Genet., 16:391-395 (1975)). Subsequent studies, however, failed to consistently replicate such results (Surti et al., Am.

J. Hum. Gene., 47:635-643 (1990); Carritt et al., Proc. Natl. Acad. Sci. USA, 79:7400-7404 (1982); Parrington et al., J. Med. Genet., 21:1-12 (1984); Deka et al., Am. J. Hum. Genet., 47:644-655 (1990); Dahl et al, Cancer Genet. Cytogenet., 46:115-123 (1990)).

7. In section III, titled **Summary Of The Invention** please replace page 10, 1st complete paragraph with the following:

It is another object of the invention to provide homozygous stem cells (HS) derived from blastocyst-like masses mitotically created by: (a) fusing two oocytes or two spermatids; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte. Additionally, screening for stem cells that are homozygous is performed using genotyping when methods (a) or (d) are used.

8. In section III, titled **Summary Of The Invention** please replace page 10, 2nd complete paragraph with the following:

It is also an object of the invention to provide methods of deriving homozygous stem cells from non-fertilized post-meiosis I diploid germ cells. Preferably, HS cells are derived using methods for preventing the extrusion of the second polar body from an oocyte during oogenesis, or allowing the extrusion of the second polar body and spontaneous genomic self-replication under appropriate conditions of such haploid oocyte to create a blastocyst-like mass from which HS cells are isolated.

9. In section III, titled **Summary Of The Invention** please replace page 10, 5th complete paragraph with the following:

Illustrative types of epithelial cells include but are not limited to keratinizing epithelial cells; wet-stratified barrier epithelia; lining epithelial cells; exocrine-secreting epithelial cells; endocrine-secreting epithelial cells; extracellular matrix-secreting epithelial cells; absorptive epithelial cells, such as those of the gut, exocrine glands, and urogenital tract; and contractile epithelial cells. Illustrative types of connective tissue

cells include but are not limited to extracellular matrix-secreting cells; cells specialized for metabolism and storage; and circulating cells of the blood and immune systems. Illustrative types of muscle cells include but are not limited to contractile cells and ciliated cells with propulsive function. Illustrative types of nervous or sensory cells include but are not limited to: a) sensory transducers; b) autonomic neurons; c) supporting cells of sense organs; d) peripheral neurons; and, e)neurons and glial cells of central nervous system. Illustrative types of reproductive cells include but are not limited to germ cells and nurse cells.

10. In section IV, titled **Brief Description of the Figures** please replace page 13, Figure 5D with the following:

Figure 5D: Photograph of the morphology of lymphocytes derived from mouse HS cells.

11. In section IV, titled **Brief Description of the Figures** please replace page 14, Figure 8D with the following:

Figure 8D: Photograph of an isolated inner cell mass growing on feeder layers derived from human homozygous post-meiosis I diploid oocytes.

12. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 15, 5th complete paragraph with the following:

Progenitor cell lines, each capable of producing cells from one of the three germ layers, i.e. the endoderm, mesoderm and ectoderm, are referred to in the present application as "multi-potent". While each progenitor cell line is not terminally differentiated and can continue to divide for the lifetime of an animal, it is considered to be committed to different tissues or cells from only one type of embryonic layer. Therefore, particular progenitor cell lines may be differentiated into bone, cartilage, smooth muscle, striated muscle and hematopoietic cells (mesoderm); liver, primitive gut, and respiratory epithelium (endoderm); or, neurons, glial cells, hair follicles and tooth buds (ectoderm). The term "progenitor cells" hence may be used synonymously with

"multi-potent stem cells" or "precursor cells". Such progenitor cell lines, which are created by the directed differentiation of HS cells *in vivo* (where the term "*in vivo*" includes differentiation induced by encapsulating said HS cells in an isogenic or allogeneic animal to generate stemplasms from such encapsulated cells) or *in vitro*, can be maintained in culture as permanent cell lines.

13. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 16, 4th complete paragraph with the following:

A "homozygous stem cell", previously termed a "teratoma stem cell" or a "TS cell", is an undifferentiated stem cell arising from a non-fertilized post-meiosis I diploid germ cell. Preferably, it is formed by preventing the extrusion of the second polar body during oogenesis (or "activation"), or allowing the extrusion of the second polar body and spontaneous genomic self-replication of the haploid oocyte in appropriate conditions. Homozygous stem (HS) cells are isolated cells generated from the inner cell mass of blastocyst-like masses that develop upon "mitotic activation" of non-fertilized post-meiosis I diploid germ cells, which can be accomplished by: (a) fusing two oocytes or two spermatids; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte. Additionally, screening for stem cells that are homozygous is performed using genotyping when methods (a) or (d) are used.

14. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 17, 3rd full paragraph with the following:

As used herein, the term "mitotically activated" means acquiring the ability to undergo regular cell divisions mitotically, and includes both parthenogenetic activation of oocytes and androgenetic activation of spermatids.

15. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 18, 3rd complete paragraph with the following:

For example, Hole, Cells Tissues Organs, **165**: 181-189 (1999), incorporated by reference herein describes methods for directing the differentiation of hematopoietic cells from embryonic stem cells in vitro. In addition, Doetschman et al., Embryol. Exp. Morphol., 87: 27-45 (1985), incorporated by reference herein, suggest that the withdrawal of leukemia inhibitory factor (LIF) from ES cells grown in suspended culture results in the formation of cystic embryoid bodies containing blood islands made up of erythrocytes and macrophages. The production of other hematopoietic cells, including neutrophils, mast cells, macrophages and erythroid cells, from stem cells has also been described. (See, e.g., Wiles and Keller, Development, **111**: 259-267 (1991); Keller et al, Mol. Cell. Biol. **13**: 473-486 (1993a); and Lieschke and Dunn, Exp. Hematol., **23**: 328-334 (1995), each of which are hereby incorporated by reference herein in their entirety). Such methods are applicable to HS cells of the present invention.

16. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 19, 1st complete paragraph with the following:

Techniques for eliciting the differentiation of stem cells into a variety of neuronal cells are described by Okabe et al. Mech. Dev., **59**: 89-102 (1996), incorporated by reference herein. Likewise, McDonald et al., Nature Medicine, **5**:1410-1412 (1999), incorporated by reference herein, describe oligodendrocytes and neurons derived from stem cells that have particular use in treating injured spinal cords. These techniques can be used with HS cells of the present invention.

17. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 22, 1st complete paragraph with the following:

HS cells are created by: (a) fusing two oocytes or two spermatids followed by screening for homozygous stem cells by genotyping; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte followed by screening for homozygous stem cells by genotyping. Figure 2 provides a schematic representation of

spermatogenesis and oogenesis, showing the difference in phases of mitosis and meiosis in males and females.

18. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 23, 1st complete paragraph with the following:

Polyethylene glycol has also been shown to induce fusion of ovulated oocytes (see, e.g., GG Sekirina, Ontogenez, **16**(6):583-8 (1985), and Gulyas BJ, Dev. Biol. **101**(1):246-50 (1984), incorporated by reference herein). Alternatively, Nogues et al., Zygote, **2**(1):15-28 (1994), incorporated by reference herein, describes the induction of oocyte fusion by inactivated Sendai virus, resulting in the production of "zygotes" or "oocyte fusion products (OFP)" that are able to undergo the first stages of embryonic development. For a review of oocyte fusion techniques, see Gulyas BJ, Dev. Biol., **4**:57-80 (1986), incorporated by reference herein. For a detailed protocol for fusion of mouse oocytes, see Hogan et al. supra, pp. 148-150, wherein harvested eggs with their cumulus cells attached are maintained in a solution of 7% ethanol in Dulbecco's PBS for 5 minutes, washed with medium, and incubated at 37°C for 5 hours. The cumulus cells are subsequently removed by treatment with hyaluronidase. Figure 3 provides a depiction of the fusion of oocytes and the development of oocyte fusion products.

19. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 42, 3rd complete paragraph with the following:

Gene trapping can be used to identify genes likely to be involved in early hematopoietic commitment. In this strategy, genes are mutagenized at random by the insertion of a reporter construct into the genome of HS cells, often coupled to an expression construct conferring drug resistance. Typically, the expression profile of the "trapped" gene is then observed following production of chimeric animals; candidate genes can then be identified by sequencing. An alternative approach is to use *in vitro* differentiation of HS cells as a prescreen. Using the OP9-dependent model of *in vitro* ES cell hematopoietic differentiation, expression trapping of hematopoietic and endothelial cells has been demonstrated (Stanford et al., Blood **92**:4622-4631 (1998)).

20. In section V, titled **Detailed Description Of The Preferred Embodiments** t please replace page 43, 2nd complete paragraph with the following:

The-BM stromal cell line, OP9, is cultured as a monolayer in αMEM supplemented with 2.2 g/liter sodium bicarbonate and 20% FCS (ES grade and lot tested; Cyclone, Logan, UT). OP9 media is also used for HS/OP9 co cultures. HS cells are cultured on a confluent monolayer of mitomycin C-treated embryonic fibroblasts with 1 ng/ml leukemia inhibitory factor (R & D Systems, Minneapolis, MN). HS and embryonic fibroblast cells are maintained in DMEM, supplemented with 15% FCS, 2 mM glutamine, 110 μg/ml sodium pyruvate, 50 μM2-mercaptoethanol, and 10 mM Hepes (pH 7.4). All co-cultures are incubated at 37°C in a humidified incubator containing 5% CO2 in air. Periodic testing indicates that all cell lines were maintained as mycoplasma-free cultures.

21. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 43, 3rd complete paragraph with the following:

For hematopoietic induction, a single-cell suspension of HS cells is seeded onto a confluent OP9 monolayer in 6-well plates. The media is changed at day 3; by day 5, nearly 100% of the HS colonies differentiate into mesoderm-like colonies. The cocultures are trypsinized (0.25%; GIBCO/BRL) at day 5; the single-cell suspension is preplated for 30 min; and nonadherent cells (1 to 2 x 106) are reseeded onto new confluent OP9 layers in 10-cm dishes. At day 6 or day 7, small clusters of hematopoietic-like, smooth round cells begin to appear. At day 8, loosely adherent cells are gently washed off and placed onto new OP9 layers (without trypsin). This treatment enriches cells with hematopoietic potential and leaves behind differentiated mesoderm and undifferentiated HS colonies.

22. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 44, 2nd complete paragraph with the following:

To generate transformed cell lines, IL- 7 (5 ng/ml) (R & D Systems) is added at

day 8 to Flt-3L-containing HS/OP9 co-cultures to maintain immature pre-B Cells-Co-cultures are infected by adding an undiluted virus stock harvested from a 4-day confluent plate of the producer cell line. Co-cultures from a 10-cm dish are infected by replacing the medium with 3 ml of virus stock containing 4 μg/ml of polybrene (Sigma) and IL- 7. The plate is rocked periodically at 37°C for 2 to 4 hours. After this period, 5 ml of fresh OP9 medium containing IL- 7 is added to the plate. The medium is changed 5 days later to medium with IL- 7, but without Flt-3L. Subsequent media changes lack IL- 7. Flow cytometry analyses show that all transformed lines display the same phenotype. In each experiment a significant population of CD45R+ CD24+ IgMe immature pre-B cells are present. Infected cells are grown in bulk, and then cloned by limiting dilution. The presence of integrated copies of the viral genome is confirmed by Southern blot analysis.

23. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 45, 1st complete paragraph with the following:

Flt-3L is added at day 5 of the HS/OP9 co-culture, when hematopoietic cells are first observed. Analysis of the day 19 co-cultures reveals that the addition of Flt-3L dramatically enhances the generation of B lymphocytes from the HS/OP9 co-cultures (60~/o vs. 6% CD45R+ cells, with Flt-3L and without Flt-3L, respectively). Thus, the addition of Flt-3L to the HS/OP9 co-culture at day 5 increases the recovery of B lineage cells at later times by ~ ten-fold. Significantly, the frequency of myeloid, CD 11 b $^{+}$ (Mac-1), and erythroid, TER -119, cells is diminished in the Fl t- 3 L-treated cultures. Evidence for T lymphocyte differentiation is not observed in these cultures. The phenotype of day 19 HS/OP9 co-culture cells clearly shows that the addition of Flt-3L results in a specific increase in the generation of CD^{19}. CD45R- AA4.1- CD^{24}+ IgM~ cells, although one observes only a slight increase in the total number of cells (~30%). With the addition of Flt-3L at day 5, B lymphopoiesis in the HS/OP9 co-culture system occurs with high efficiency.

REMARKS

It is respectfully requested that the Examiner enter these amendments prior to examining the application on its merits. These amendments are being made to correct grammatical and/or typographical errors. This amendment contains no new matter.

Respectfully submitted,

SHANKS & HERBERT

By: _____
Toni-Junell Herbert
Reg. No. 34, 348

Date: 1/4/02

TransPotomac Plaza
1033 N. Fairfax Street
Suite 306
Alexandria, VA 22314
(703) 683-3600

MARKED UP VERSION OF THE SPECIFICATION

IN THE SPECIFICATION:

1. In section II, titled **Background of the Invention and Description of Related Art**, please replace page 2, 3rd complete paragraph with the following:

In 1981, Evans and Kaufman described the technique for isolating embryonic stem (ES) cell lines from mouse blastocysts. Evans et al., "*Establishment in Culture of Pluripotent Cells from Mouse Embryos,*" Nature **292**:154-6 (1981). In this procedure, the inner cell mass (ICM) was used to give rise to a cell line that remained undifferentiated and pluripotent, i.e., the cells had the capacity to develop into any cell type. ES cell lines were subsequently produced in other animal models including chicken (Pain et al, Development **122**:2339-48 (1996)), hamster (Doetschmann et al., Dev. Biol. **127**:224-7 (1988)), swine (Wheeler et al., Reprod. Fertil. Dev. **6**:563-8 (1994)), marmoset (Thompson et al., Biol. Reprod. **55**:254-9 (1996)), and rhesus monkey (Thompson et al., Proc. Natl. Acad. Sci. USA **92**:7844-8 (1995)).

2. In section II, titled **Background of the Invention and Description of Related Art** please replace page 5, 4th complete paragraph with the following:

The development of other sources of pluripotent cells is hence needed in the art. The present invention provides one such source. In one embodiment, the present invention provides isolated homozygous stem (HS) cells that are isolated from a blastocyst-like mass that is created by: (a) fusing two oocytes or two spermatids; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte. Additionally, screening for stem cells that are homozygous is performed using genotyping when methods (a) or (d) are used.

3. In section II, titled **Background of the Invention and Description of Related Art** please replace page 6, 1st complete paragraph with the following:

The HS cells of the present invention are pluripotent, and raise no ethical concerns as they are isolated from cell-masses that are non-fertilized, and incapable of developing into viable embryos. Moreover, immunohistocompatibility matching is difficult to accomplish when heterozygous ES cell lines are employed in tissue or cell transplantation therapy, or maintained in banks and/or depositories. This is because the ES cell lines, including those developed by Advanced Cell Technology and other organizations, are derived from fertilized embryos or from nuclear transfer techniques using adult differentiated cells, and are genomically heterozygous. Because the pluripotent stem cells of the present invention are homozygous (with minimal heterozygosity or uniform homozygosity), such cells may be used to overcome immunohistocompatibility problems faced by currently available transplantation, cell replacement, and gene therapy techniques employing ES cell lines, or maintaining ES cell line banks and/or depositories.

4. In section II, titled **Background of the Invention and Description of Related Art** please replace page 6, 3rd complete paragraph with the followoing:

Upon proper activation, a metaphase II oocyte can proceed to complete meiosis by the extrusion of one [of] chromatid (i.e. the secondary polar body) and give rise to a haploid cell. Such meiosis-completed haploid oocyte self-replicates without cytokinesis, rendering it diploid and uniformly homozygous. Such meiosis-completed haploid oocytes, hence, may also be used to create the homozygous stem cells of the present invention with no heterozygosity. See also, Kaufman M.H., Robertson E.J., Handyside A.H., Evans M.J., "*Establishment of pluripotential cell lines from haploid mouse embryos*," J. Embryol. Exp. Morphol., 73:249-61 (1983).

5. In section II, titled **Background of the Invention and Description of Related Art** please replace page 8, 2nd complete paragraph with the following:

Naturally occurring spontaneous teratomas are diploid and occasionally polyploid (Surti *et al.,* Am. J. Hum. Gene. 47:635-643 (1990)). It is believed that diploid teratomous tissue occurs secondary to meiosis I, or due to fusion of the second polar body with the ovum (Eppig and Eicher, Genetics, 103:797-812 (1 983); Eppig and Eicher, J. Hered., 79:425-429 (1988)). Further, teratomas have been prove[d]n to be genetically

homozygous in heterozygous hosts (Linder, Proc. Natl. Acad. Sci. USA, 63:699-704 (1969); Linder and Power, Ann. Hum. Genet. 34:21-30, (1970); Linder et al., Nature, 254:597-598 (1975); Kaiser-McCaw et al., Cytogenet. Cell. Genet., 16:391-395 (1975)). Subsequent studies, however, failed to consistently replicate such results (Surti et al., Am. J. Hum. Gene., 47:635-643 (1990); Carritt et al., Proc. Natl. Acad. Sci. USA, 79:7400-7404 (1982); Parrington et al., J. Med. Genet., 21:1-12 (1984); Deka et al., Am. J. Hum. Genet., 47:644-655 (1990); Dahl et al, Cancer Genet. Cytogenet., 46:115-123 (1990)).

6. In section III, titled **Summary Of The Invention** please replace page 10, 1st complete paragraph with the following:

It is another object of the invention to provide homozygous stem cells (HS) derived from blastocyst-like masses mitotically created by: (a) fusing two oocytes or two spermatids; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte. Additionally, screening for stem cells that are homozygous is performed using genotyping when methods (a) or (d) are used.

7. In section III, titled **Summary Of The Invention** please replace page 10, 2nd complete paragraph with the following:

It is also an object of the invention to provide methods of deriving homozygous stem cells from non-fertilized post-meiosis I diploid germ cells. Preferably, HS cells are derived using methods for preventing the extrusion of the second polar body from an oocyte during oogenesis, or allowing the extrusion of the second polar body and spontaneous genomic self-replication under appropriate conditions of such haploid oocyte to create a blastocyst-like mass from which HS cells are [extracted] isolated.

8. In section III, titled **Summary Of The Invention** please replace page 10, 5th complete paragraph with the following:

Illustrative types of epithelial cells include but are not limited to keratinizing epithelial cells; wet-stratified barrier epithelia; lining epithelial cells; exocrine-secreting epithelial cells; endocrine-secreting epithelial cells; extracellular matrix-secreting epithelial cells; absorptive epithelial cells, such as those of the gut, exocrine glands, and urogenital

tract; and contractile epithelial cells. Illustrative types of connective tissue cells include but are not limited to extracellular matrix-secreting cells; cells specialized for metabolism and storage; and circulating cells of the blood and immune systems. Illustrative types of muscle cells include but are not limited to contractile cells and ciliated cells with propulsive function. Illustrative types of nervous or sensory cells include but are not limited to: a) sensory transducers; b) autonomic neurons; c) supporting cells of sense organs; [and] d) peripheral neurons; and, e) neurons and glial cells of central nervous system. Illustrative types of reproductive cells include but are not limited to germ cells and nurse cells.

9. In section IV, titled **Brief Description of the Figures** please replace page 13, Figure 5D with the following:

Figure 5D: Photograph of the morphology of lymphocytes derived from mouse HS cells.

10. In section IV, titled **Brief Description of the Figures** please replace page 14, Figure 8D with the following:

Figure 8D: Photograph of an isolated inner cell mass growing on feeder layers [(D)] derived from human homozygous post-meiosis I diploid oocytes.

11. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 15, 5th complete paragraph with the following:

Progenitor cell lines, each capable of producing cells from one of the three germ layers, i.e. the endoderm, mesoderm and ectoderm, are referred to in the present application as "multi-potent". While each progenitor cell line is not terminally differentiated and can continue to divide for the lifetime of an animal, it is considered to be committed to different tissues or cells from only one type of embryonic layer. Therefore, particular progenitor cell lines may be differentiated into bone, cartilage, smooth muscle, striated muscle and hematopoietic cells (mesoderm); liver, primitive gut, and respiratory epithelium (endoderm); or, neurons, glial cells, hair follicles and tooth buds (ectoderm). The term "progenitor cells" hence may be used synonymously with "multi-potent stem cells" or "precursor cells". Such progenitor cell[s] lines, which are created by the directed

differentiation of HS cells *in vivo* (where the term "*in vivo*" includes differentiation induced by encapsulating said HS cells in an isogenic or allogeneic animal to generate stemplasms from such encapsulated cells) or *in vitro*, can be maintained in culture as permanent cell lines.

12. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 16, 4th complete paragraph with the following:

A "homozygous stem cell", previously termed a "teratoma stem cell" or a "TS cell", is an undifferentiated stem cell arising from a non-fertilized post-meiosis I diploid germ cell. Preferably, it is formed by preventing the extrusion of the second polar body during oogenesis (or "activation"), or allowing the extrusion of the second polar body and spontaneous genomic self-replication of the haploid oocyte in appropriate conditions. Homozygous stem (HS) cells are isolated cells generated from the inner cell mass of blastocyst-like masses that develop upon "mitotic activation" of non-fertilized post-meiosis I diploid germ cells, which can be accomplished by: (a) fusing two oocytes or two spermatids; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte. Additionally, screening for stem cells that are homozygous is performed using genotyping when methods (a) or (d) are used.

13. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 17, 3rd full paragraph with the following:

As used herein, the term "mitotically activated" means acquiring the ability to undergo regular cell divisions mitotically, and includes both parthenogenetic activation of oocytes and androgenetic activation of spermatids. [For the purposes of this application, mitotically activated is used synonymously with parthenogenic activation or androgenetic activation.]

14. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 18, 3rd complete paragraph with the following:

For example, Hole, Cells Tissues Organs, **165**: 181-189 (1999), incorporated by reference herein[)] describes methods for directing the differentiation of hematopoietic

cells from embryonic stem cells in vitro. In addition, Doetschman et al., Embryol. Exp. Morphol., 87: 27-45 (1985), incorporated by reference herein[)], suggest that the withdrawal of leukemia inhibitory factor (LIF) from ES cells grown in suspended culture results in the formation of cystic embryoid bodies containing blood islands made up of erythrocytes and macrophages. The production of other hematopoietic cells, including neutrophils, mast cells, macrophages and erythroid cells, from stem cells has also been described. (See, e.g., Wiles and Keller, Development, **111**: 259-267 (1991); Keller et al, Mol. Cell. Biol. **13**: 473-486 (1993a); and Lieschke and Dunn, Exp. Hematol., **23**: 328-334 (1995), each of which are hereby incorporated by reference herein in their entirety). Such methods are applicable to HS cells of the present invention.

15. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 19, 1st complete paragraph with the following:

Techniques for eliciting the differentiation of stem cells into a variety of neuronal cells are described by Okabe et al. Mech. Dev., **59**: 89-102 (1996), incorporated by reference herein[)]. Likewise, McDonald et al., Nature Medicine, **5**:1410-1412 (1999), incorporated by reference herein, describe oligodendrocytes and neurons derived from stem cells that have particular use in treating injured spinal cords. These techniques can be used with HS cells of the present invention.

16. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 22, 1st complete paragraph with the following:

HS cells are created by: (a) fusing two oocytes or two spermatids followed by screening for homozygous stem cells by genotyping; (b) preventing the extrusion of the second polar body during oogenesis; (c) allowing the extrusion of the second polar body and spontaneous genomic self-replication in appropriate conditions; or, (d) transferring two haploid egg or sperm nuclei into an enucleated oocyte followed by screening for homozygous stem cells by genotyping. Figure 2 provides a schematic representation of spermatogenesis and oogenesis, showing the difference in phases of mitosis and meiosis in males and females.

17. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 23, 1st complete paragraph with the following:

Polyethylene glycol has also been shown to induce fusion of ovulated oocytes (see, e.g., GG Sekirina, Ontogenez, **16**(6):583-8 (1985), and Gulyas BJ, Dev. Biol. **101**(1):246-50 (1984), incorporated by reference herein). Alternatively, Nogues et al., Zygote, **2**(1):15-28 (1994), incorporated by reference herein[])], describes the induction of oocyte fusion by inactivated Sendai virus, resulting in the production of "zygotes" or "oocyte fusion products (OFP)" that are able to undergo the first stages of embryonic development. For a review of oocyte fusion techniques, see Gulyas BJ, Dev. Biol., **4**:57-80 (1986), incorporated by reference herein. For a detailed protocol for fusion of mouse oocytes, see Hogan et al. supra, pp. 148-150, wherein harvested eggs with their cumulus cells attached are maintained in a solution of 7% ethanol in Dulbecco's PBS for 5 minutes, washed with medium, and incubated at 37°C for 5 hours. The cumulus cells are subsequently removed by treatment with hyaluronidase. Figure 3 provides a depiction of the fusion of oocytes and the development of oocyte fusion products.

18. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 42, 3rd complete paragraph with the following:

Gene trapping can be used to identify genes likely to be involved in early hematopoietic commitment. In this strategy, genes are mutagenized at random by the insertion of a reporter construct into the genome of HS cells, often coupled to an expression construct conferring drug resistance. Typically, the expression profile of the "trapped" gene is then observed following production of chimeric animals; candidate genes can then be identified by sequencing. An alternative approach is to use *in vitro* differentiation of H[T]S cells as a prescreen. Using the OP9-dependent model of *in vitro* ES cell hematopoietic differentiation, expression trapping of hematopoietic and endothelial cells has been demonstrated (Stanford et al., Blood **92**:4622-4631 (1998)).

19. In section V, titled **Detailed Description Of The Preferred Embodiments** t please replace page 43, 2nd complete paragraph with the following:

The-BM stromal cell line, OP9, is cultured as a monolayer in αMEM supplemented with 2.2 g/liter sodium bicarbonate and 20% FCS (ES grade and lot tested; Cyclone, Logan, UT). OP9 media is also used for H[T]S/OP9 co cultures. HS cells are cultured on a confluent monolayer of mitomycin C-treated embryonic fibroblasts with 1 ng/ml leukemia inhibitory factor (R & D Systems, Minneapolis, MN).

HS and embryonic fibroblast cells are maintained in DMEM, supplemented with 15% FCS, 2 mM glutamine, 110 μg/ml sodium pyruvate, 50 μM2-mercaptoethanol, and 10 mM Hepes (pH 7.4). All co-cultures are incubated at 37°C in a humidified incubator containing 5% CO2 in air. Periodic testing indicates that all cell lines were maintained as mycoplasma-free cultures.

20. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 43, 3rd complete paragraph with the following:

For hematopoietic induction, a single-cell suspension of HS cells is seeded onto a confluent OP9 monolayer in 6-well plates. The media is changed at day 3; by day 5, nearly 100% of the H[T]S colonies differentiate into mesoderm-like colonies. The cocultures are trypsinized (0.25%; GIBCO/BRL) at day 5; the single-cell suspension is preplated for 30 min; and nonadherent cells (1 to 2 x 106) are reseeded onto new confluent OP9 layers in 10-cm dishes. At day 6 or day 7, small clusters of hematopoietic-like, smooth round cells begin to appear. At day 8, loosely adherent cells are gently washed off and placed onto new OP9 layers (without trypsin). This treatment enriches cells with hematopoietic potential and leaves behind differentiated mesoderm and undifferentiated HS colonies.

21. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 44, 2nd complete paragraph with the following:

To generate transformed cell lines, IL- 7 (5 ng/ml) (R & D Systems) is added at day 8 to Flt-3L-containing H[T]S/OP9 co-cultures to maintain immature pre-B Cells-Co-cultures are infected by adding an undiluted virus stock harvested from a 4-day confluent plate of the producer cell line. Co-cultures from a 10-cm dish are infected by replacing the medium with 3 ml of virus stock containing 4 μg/ml of polybrene (Sigma) and IL- 7. The plate is rocked periodically at 37°C for 2 to 4 hours. After this period, 5 ml of fresh OP9 medium containing IL- 7 is added to the plate. The medium is changed 5 days later to medium with IL- 7, but without Flt-3L. Subsequent media changes lack IL- 7. Flow cytometry analyses show that all transformed lines display the same phenotype. In each experiment a significant population of CD45R+ CD24+ IgMe immature pre-B cells are present. Infected cells are grown in bulk, and then cloned by limiting dilution. The presence of integrated copies of the viral genome is confirmed by Southern blot analysis.

22. In section V, titled **Detailed Description Of The Preferred Embodiments** please replace page 45, 1st complete paragraph with the following:

Flt-3L is added at day 5 of the H[T]S/OP9 co-culture, when hematopoietic cells are first observed. Analysis of the day 19 co-cultures reveals that the addition of Flt-3L dramatically enhances the generation of B lymphocytes from the HS/OP9 co-cultures (60~/o vs. 6% CD45R+ cells, with Flt-3L and without Flt-3L, respectively). Thus, the addition of Flt-3L to the HS/OP9 co-culture at day 5 increases the recovery of B lineage cells at later times by ~ ten-fold. Significantly, the frequency of myeloid, CD 11 b^+ (Mac-1), and erythroid, TER -119, cells is diminished in the Fl t- 3 L-treated cultures. Evidence for T lymphocyte differentiation is not observed in these cultures. The phenotype of day 19 HS/OP9 co-culture cells clearly shows that the addition of Flt-3L results in a specific increase in the generation of CD^{19}. CD45R- AA4.1- CD^{24}+ IgM~ cells, although one observes only a slight increase in the total number of cells (~30%). With the addition of Flt-3L at day 5, B lymphopoiesis in the HS/OP9 co-culture system occurs with high efficiency.